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Interleukin-6 is increased in plasma and cerebrospinal fluid of community-dwelling domestic dogs with acute ischaemic stroke

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Inflammatory cytokines are potential modulators of infarct progression in acute ischaemic stroke, and are therefore possible targets for future treatment strategies. Cytokine studies in animal models of surgically induced stroke may, however, be influenced by the fact that the surgical intervention itself contributes towards the cytokine response. Community-dwelling domestic dogs suffer from spontaneous ischaemic stroke, and therefore, offer the opportunity to study the cytokine response in a noninvasive set-up. The aims of this study were to investigate cytokine concentrations in plasma and cerebrospinal fluid (CSF) in dogs with acute ischaemic stroke and to search for correlations between infarct volume and cytokine concentrations. Blood and CSF were collected from dogs less than 72 h after a spontaneous ischaemic stroke. Infarct volumes were estimated on MRIs. Interleukin (IL)-2, IL-6, IL-8, IL-10 and tumour necrosis factor in the plasma, CSF and brain homogenates were measured using a canine-specific multiplex immunoassay. IL-6 was significantly increased in plasma ($P = 0.04$) and CSF ($P = 0.04$) in stroke dogs compared with healthy controls. The concentrations of other cytokines, such as tumour necrosis factor and IL-2, were unchanged. Plasma IL-8 levels correlated significantly with infarct volume (Spearman's $r = 0.8$, $P = 0.013$). The findings showed increased concentrations of IL-6 in the plasma and CSF of dogs with acute ischaemic stroke comparable to humans. We believe that dogs with spontaneous stroke offer a unique, noninvasive means of studying the inflammatory processes that accompany stroke while reducing confounds that are unavoidable in experimental models. *NeuroReport* **28:134–140 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Adverse inflammatory reactions have been shown to play key roles in the progressive pathology and loss of neurological function in acute ischaemic stroke [1]. Inflammatory cytokines, in particular interleukin (IL)-1β, IL-6, IL-10 and tumour necrosis factor (TNF), are potential modulators of infarct progression [2,3]. Accordingly, these cytokines may represent possible targets for future neuroprotective strategies.

Several experimental studies have sought to clarify the inflammatory roles of these cytokines after stroke. It is clear that IL-1β can play both a neurotoxic and a proinflammatory role [4,5], whereas IL-10 is believed to behave primarily as an anti-inflammatory neuroprotective cytokine [6]. For IL-6 and TNF, the effect of elevated expression is less straightforward as these cytokines seem to possess both neurotoxic and neuroprotective properties [3,7,8]. IL-2, although usually not considered a main player in ischaemic stroke, seems to contribute towards the adverse inflammatory reactions [9].

To date, experimental rodent models of ischaemic stroke have highlighted a role for mediators of inflammation in acute ischaemic stroke [10]. However, the translation of otherwise encouraging results from animal models into humans has been disappointing [11]. Apart from early (< 4.5 h) intravenous administration of recombinant tissue plasminogen activators, treatment strategies have

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failed to prevent progressive cell death of the peri-infarct area in humans [12]. Even though rodent models have been developed that enable control of the size of the cerebral injury and provide an opportunity to study mechanistic pathways, they often fail to reproduce the heterogeneity of human stroke patients, where disease is multifactorial and infarcts of various sizes appear at different sites [10]. With respect to studies of inflammatory reactions in animal models of surgically induced stroke, interpretation of results is further complicated by the fact that the surgical intervention contributes towards the inflammatory response [13].

Although less common than in humans, domestic dogs also suffer from naturally occurring ischaemic stroke [14,15]. Affected dogs are presented at veterinary hospitals where the presentation of acute neurological signs and MRI findings resemble those in humans [14,15]. As the anatomy of dogs also shares many similarities with humans in respect to the gyrencephalic brain, grey–white matter composition and circle of Willis [16,17], dogs with stroke could provide a highly translatable noninvasive clinical model for early infarct development. Supposedly, inflammatory mechanisms in canine ischaemic stroke are also comparable to humans, which would further support the use of this model. However, poststroke inflammation including the expression of cytokines with acute ischaemic stroke has only been subject to limited investigation in dogs [18].

In the present study, we investigated the poststroke concentrations of the cytokines IL-2, IL-6, IL-8, IL-10 and TNF in plasma and cerebrospinal fluid (CSF) in community-dwelling domestic dogs with spontaneous ischaemic stroke compared with healthy control dogs. The aim of this study was to investigate the inflammatory cytokine response after spontaneous stroke in dogs as a potential model for early inflammatory mechanisms in ischaemic stroke.

Methods

Animals

Nine community-dwelling domestic dogs of various breeds presenting with acute neurological signs (< 72 h) and a MRI diagnosis of ischaemic stroke were investigated in this prospective multicentre study including three veterinary referral hospitals [median age: 9 years; interquartile range (IQR) = 5.5–11.5] (Table 1).

Control blood samples were collected at the University Hospital for Companion Animals (UHCA), Copenhagen University, Denmark, from nine healthy dogs (five females and four males) with a median age of 5 years (IQR = 2.5–8.5); dogs of various breeds (three Greyhounds, two Labrador retrievers, two Dachshunds, one Whippet and one Italian Greyhound) were enroled as controls at the UHCA. Control CSF samples were collected from three healthy large mixed breeds (two females, one male; median age: 5 years; IQR = 3–12 years) that were euthanized at the UHCA at the owners' request with no apparent neurological or systemic conditions.

The study was approved by the Local Administrative and Ethics Committee, Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen (permission number: 1N/2013), and all procedures were completed with the owners' consent.

Magnetic resonance imaging

MRIs were performed under general anaesthesia [premedication with methadone (0.2–0.3 mg/kg) intramuscularly or dexdomitor (41 mcg/m^2) and butorphanol (0.1 mg/kg) intravenously, induction of anaesthesia with diazepam (0.2–0.3 mg/kg) intravenously, followed by propofol intravenous dosed to allow endotracheal intubation and anaesthesia maintained by isoflurane in oxygen administered by inhalation]. The following MRI equipment was used: 0.2 T (Esaote Vet MR; Esaote, Genoa, Italy) at the UHCA, 0.4 T (Aperto Permanent Magnet; Hitachi, Tokyo, Japan) at Davies Veterinary Specialists and 1.5 T MRI (Siemens Symphony, Erlangen, Germany) at Fitzpatrick's Referrals, UK. MRI included T1- and T2-weighted images, transverse and sagittal sections and contrast images, and fluid-attenuated inversion recovery. Gadolinium dimeglumine (Magnevist; Schering Diagnostics AG, Berlin, Germany), gadoteridol (Prohance; Bracco Imaging, Cranberry, New Jersey, USA) or gadoteric acid (Dotarem; Guerbet, Villepinte, France) at a dose of 0.1 mmol/kg intravenously were used as the paramagnetic contrast media.

Infarct volumetric estimations

A transparent uniform point grid was placed on the T2-weighted image transverse section images. Grid points associated with the infarct were counted for each slice, defining the region of interest (ROI). Infarct volumes were estimated using the Cavalieri theorem $V_{\text{total}} = \bar{t} \times a(p) \times \Sigma P$, where V is the total volume of the infarct, $\bar{t}$ is the average thickness between slices, $a(p)$ is the area per point and ΣP is the total number of points within the ROI [19]. Area $[a(p)]$ was adjusted between infarcts to optimize volume estimations by adjusting for differences in whole brain and infarct size (median: 12.4; minimum = 6.76; maximum = 44.89 mm^2). Infarct volumes were additionally assessed by manual perimeter tracing on each MRI slice and ROI segmentation with total volume estimation available using OsiriX Lite 6.5 (Pixmeo, Geneva, Switzerland). All volumetric estimates were carried out by a single rater, who was blinded to the patient data and cytokine results.

Table 1 Signalment, infarct location, symptomatology, approximate age of infarct and volume as estimated on MRIs by region of interest segmentation in OsiriX in nine pet dogs with spontaneous ischaemic stroke

ID	Breed	Sex	Age (years)	Infarct location	Symptomatology	Infarct age at imaging (days)	Infarct size (mm^3)	Blood	CSF	Brain biopsy
1	Rottweiler	Female	8	MCA dex	Normal mentation. Left-sided hemiparesis with absent postural reactions on left thoracic and left pelvic limbs. Absent menace response in the left eye, reduced sensorium left nasal septum and hemi-inattention left side.	< 3	4017	+	+	+
2	Mixed breed	Male[a]	5.5	Caudate nucleus sin	Normal mentation. Ataxia and leaning towards the left. Minor right-sided head tilt, ventral strabismus in the right eye.	< 3	41	+	–	–
3	Cavalier King Charles Spaniel	Male	6.5	Cerebellar midline	Depressed mentation. Initial episodes of hypertonic limbs. Right-sided head tilt, mild meiosis in the left eye, bilaterally reduced menace response, positional ventrolateral strabismus in the right eye.	< 3	120	+	+	–
4	Greyhound	Male[a]	10	RCeA dex	Depressed mentation. Left-sided torticollis, nonambulatory tetraparesis, absent menace response in the right eye, bilateral facial hypalgesia, right-sided positional ventral strabismus.	< 2	568	+	+	–
5	Greyhound	Female[a]	11	MCA dex	Depressed mentation. Left-sided hemiparesis with absent postural reactions. Decreased postural reactions on right side. Absent menace response in the left eye, and diminished sensation on the left side of the face.	< 2	5161	+	+	–
6	English Pointer	Male	13	Thalamic dex (perforantia from MCA)	Depressed mentation, general confusion, right-sided circling, decreased postural reactions on left thoracic and left pelvic limbs.	< 2	283	+	+	–
7	German Shepherd	Female	9	RCeA sin	Normal mentation. Decerebellate posture. Positional vertical nystagmus, right-sided head tilt, absent menace response in the left eye.	< 1	866	+	–	–
8	Greyhound	Male[a]	12	RCeA sin	Depressed mentation. Nonambulatory tetraparesis with decreased proprioception in all four limbs, worse on the left side. Right-sided head tilt, positional rotational nystagmus.	< 2	45	+	–	–
9	Tibetan Terrier	Male	9	RCeA sin	Normal mentation. Ataxia and hypermetric gait on the left thoracic and left pelvic limbs with mildly reduced postural reactions.	< 3	413	+	+	–

The available material from each dog is indicated by ±.
CSF, cerebrospinal fluid; dex, dextra/right; MCA, middle cerebral artery; RCeA, rostral cerebellar artery; sin, sinistra/left.
[a]Neutered.

Collection of blood and cerebrospinal fluid

All sample material was collected within 72 h of stroke onset (Table 1). Blood sampling from dogs with ischaemic stroke was performed by jugular or cephalic venous puncture using a BD vacutainer system (BD, Franklin, New Jersey, USA) connected to a 21 G butterfly catheter. Blood was collected into EDTA, serum and citrate-stabilized vacutainerCSF was collected into EDTA tubes from animals under general anaesthesia by atlanto-occipital puncture in relation to the MRI procedure.

All samples were fast frozen and stored at −80°C until further processing.

Cytokine measurements

Cytokine protein concentrations were determined in plasma and CSF (25 µl/sample) using a commercially available canine-specific multiplex immunoassay using electrochemiluminescence detection technology [MSD Canine Proinflammatory Panel 3 Ultra-Sensitive Kit (IL-2, IL-6, IL-8, TNF) and Canine IL-10 Ultra-Sensitive Kit (Mesoscale Discovery, Rockville, Maryland, USA)] and a SECTOR Imager 6000 (Mesoscale Discovery) Plate Reader according to the manufacturer's recommendations. Samples were diluted two-fold in Diluent 41 and analysed in duplex; the average value was used for statistical analyses. All samples were run at the same time at a single central laboratory.

Statistical analysis

Data were analysed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, California, USA). The Mann–Whitney *U*-test for nonparametric data was used for the comparison of medians between groups. Results are given as median values with IQR. Possible correlations between blood and CSF cytokine concentrations and correlations between infarct volumes and blood and CSF cytokine concentrations, respectively, were analysed by Spearman's rank correlation analysis for nonparametric data. *P* values less than or equal to 0.05 were considered statistically significant.

Results

Clinical presentation

Nine dogs were identified with acute neurological signs, including altered mentation, postural deficits, ataxia, cranial nerve deficits and/or vestibular signs depending on the site of the lesion (Table 1). Confirmatory MRI studies were carried out within 72 h of the clinical onset. Infarcts were located on the middle cerebral artery territory ($n=2$), the thalamus ($n=1$), the caudate nucleus ($n=1$) or the rostral cerebellar artery corresponding to the superior cerebellar artery in humans (Fig. 1 and Table 1). Eight dogs recovered after stroke, whereas one dog was euthanized on day 3 at the owners' request (Table 1, ID 1).

Infarct volumetric estimations

Infarct volumes were estimated on MRIs using the Cavalieri theorem applied to the ROIs [19] and with total volume estimation following manual tracing in OsiriX Lite 6.5 (Pixmeo), respectively. Infarct volumes estimated by these two methods were closely correlated ($r=0.98$, $P=0.0004$) (Spearman's rank correlation analysis). The largest infarcts were associated with total middle cerebral artery occlusions yielding volume estimates (OsiriX) of 4.017 and 5.161 mm^3, ID 1 and ID 5, respectively (Table 1).

Measurement of cytokine concentrations

Plasma levels of IL-6 were significantly higher in dogs with stroke (median: 6.12; IQR = 2.96–18.7) than in controls (median: 2.66; IQR = 1.14–5.3) ($P=0.04$) (Fig. 2a). Similar results were observed in CSF samples; the median IL-6 concentration in stroke dogs was 5.74 (IQR = 1.88–7.75) as opposed to 0.69 in controls (IQR = 0.04–0.93) ($P=0.04$) (Fig. 2f). The median concentrations of IL-2, IL-8, IL-10 and TNF did not differ significantly between groups in either plasma or CSF samples (Fig. 2) (Mann–Whitney *U*-test).

Correlation analyses of cytokines in plasma and cerebrospinal fluid

Cytokine concentrations in plasma did not correlate with those in CSF (IL-2 CSF vs. plasma: Spearman's $r=-0.1$, $P=0.95$; IL-6 CSF vs. plasma: Spearman's $r=-0.5$, $P=0.45$; IL-8 CSF vs. plasma: Spearman's $r=0.37$, $P=0.5$; IL-10 CSF vs. plasma: Spearman's $r=0.9$, $P=0.08$; TNF CSF vs. plasma: Spearman's $r=-0.14$, $P=0.8$).

Correlations of cytokines with infarct volume

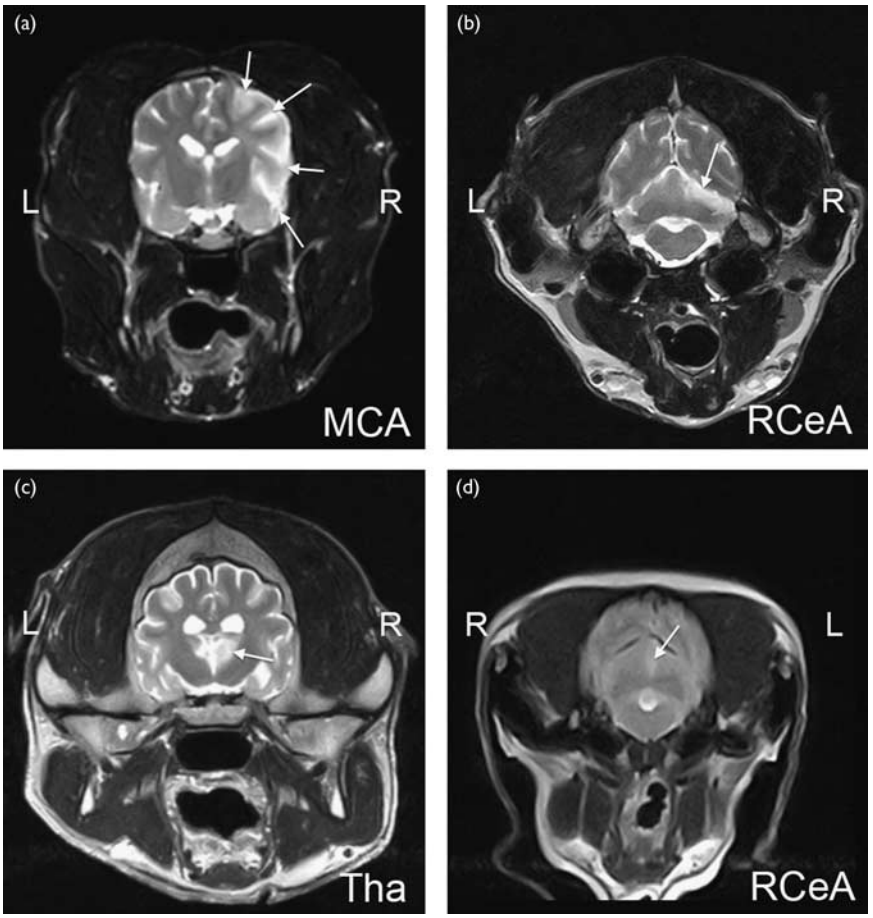
Only plasma IL-8 correlated significantly with infarct size as estimated by total volume estimation in OsiriX Lite 6.5 (Spearman's $r=0.8$, $P=0.013$). No other statistically significant correlations were found between infarct volume and cytokine concentrations in plasma or CSF (data not shown).

Discussion

The main focus of the present study was to investigate infarct size and the presence and levels of cytokines IL-2, IL-6, IL-8, IL-10 and TNF in plasma, CSF and brain tissue of community-dwelling domestic dogs within 72 h of spontaneous ischaemic stroke as a possible model of human stroke.

We found that IL-6 was significantly higher in dogs with acute ischaemic stroke of various sizes and locations, in both plasma and CSF, compared with healthy control dogs. This finding is in agreement with several studies of serum and CSF concentrations of cytokines within 72 h of human ischaemic stroke (< 72 h) [20–24]. Clinical studies in humans have shown a rapid increase in serum

Fig. 1



MRI of pet dogs with acute ischaemic stroke (arrows). (a) MRI T2-weighted image, transverse section at the level of the thalamus in an 11-year-old female Greyhound with a right-sided middle cerebral artery (MCA) infarct. (b) MRI T2-weighted image, transverse section at the level of the cerebellum in a 10-year-old male Greyhound with a right-sided rostral cerebellar artery infarct (RCeA). (c) MRI T2-weighted image, transverse section at the level of the thalamus in a 13-year-old male English Pointer with a right-sided thalamic infarct (Tha). (d) MRI T2-weighted image, transverse section at the level of the cerebellum in a 6.5-year-old male Cavalier King Charles Spaniel with a cerebellar midline infarct (RCeA).

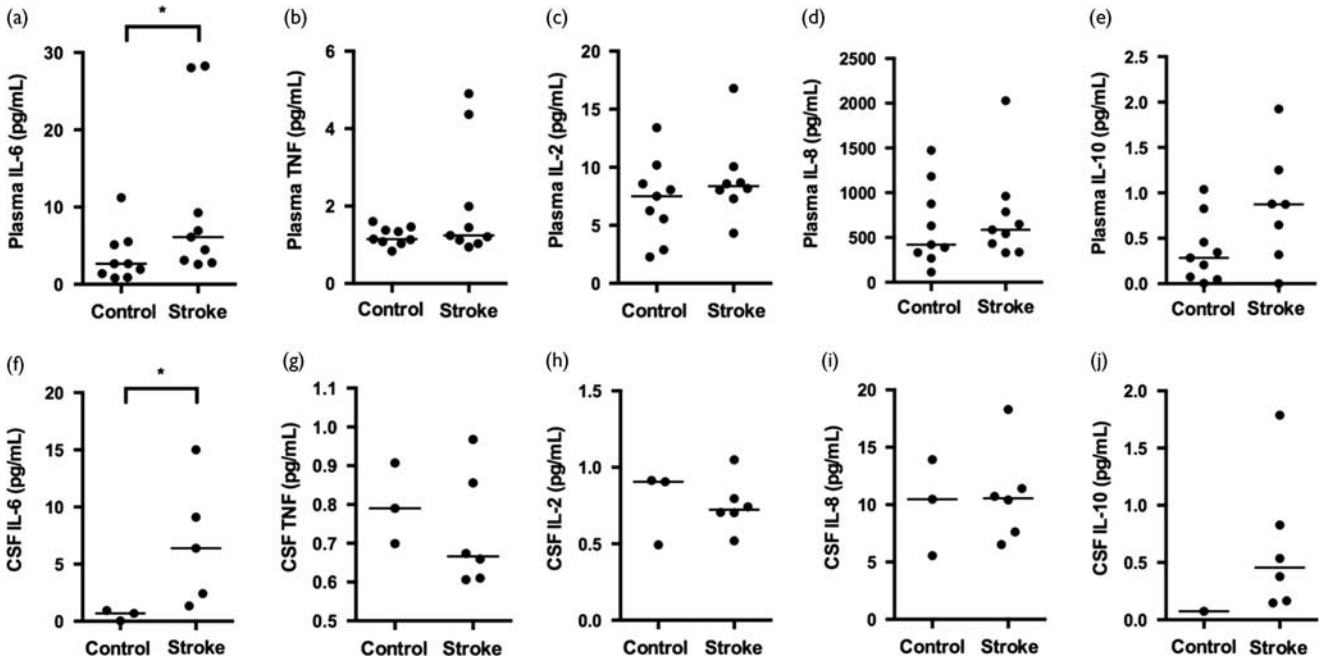
IL-6 within hours of stroke onset, reaching a plateau around day 3 and then subsiding to reach baseline levels by day 7 [22,24]. This would support that the temporal profile of IL-6 after stroke in humans and dogs is comparable. In accordance with clinical studies, experimental studies of the temporal profile of IL-6 in rodents report elevated IL-6 mRNA from 3 to 72 h, with peak expressions between 10 and 18 h [25].

IL-6 in humans in both serum and CSF has previously been shown to correlate with infarct size and stroke severity [20,22,23]. In this study, the limited sample size, heterogeneity in infarct location and time after stroke onset may in part account for our inability to verify this presently.

A difference in the median ages between stroke dogs and control dogs in the present study, although not statistically significant, should be taken into consideration as a possible confounder as IL-6 has been reported to increase with increasing age in humans as well as in the ageing rat brain in some studies [26,27]. Yet, others report no significant differences in IL-6 concentrations between young individuals and carefully screened healthy elderly individuals, and argue that a possible difference could be explained by health-related conditions in the elderly [28]. In the present study, no concurrent disease or evidence of systemic inflammation was identified in any of the stroke dogs, supporting the increase in IL-6 to be a consequence of the acute stroke condition.

We did not find any significant changes in the plasma or CSF concentrations of IL-2, IL-8, IL-10 or TNF in dogs with spontaneous stroke compared with the healthy controls. Although some human clinical studies report a parallel increase of serum TNF and IL-6 with ischaemic stroke [21,29], several others failed to detect a change in serum TNF in accordance with our results [20,24]. Investigations of TNF in CSF in humans broadly report

Fig. 2



Cytokine concentrations in dogs with acute stroke compared with healthy controls. (a–e) Plasma concentrations. (f–j) CSF concentrations. (a) IL-6 was significantly higher in stroke dogs compared with healthy controls in both plasma (median: 6.12; IQR = 2.96–18.7 vs. median: 2.66; IQR = 1.14–5.3, $P = 0.04$) and (f) CSF (median: 5.74; IQR = 1.88–7.75 vs. median: 0.69; IQR = 0.04–0.93, $P = 0.04$). Analyses of IL-2, IL-8, IL-10 and TNF did not show any significant differences between stroke dogs and controls in plasma or CSF. Data are represented as median ($n = 5$–9 per group) (Mann–Whitney *U*-test). *$P < 0.05$. CSF, cerebrospinal fluid; IL, interleukin; IQR, interquartile range; TNF, tumour necrosis factor.

elevated TNF concentrations within 24 h of stroke depending on the severity and the location of the infarct [23,29]. Experimental studies of temporal profiles of TNF mRNA in rodents also show a general peak of expression within 10–18 h of stroke onset [3,25]. In our study, samples were collected within 24 h in only one case, which could explain why we did not identify elevated TNF concentrations in our stroke dogs.

We also found no correlation between IL-6 in plasma and CSF despite the significant increase in both, which may seem counterintuitive. The limited sample size was a general weakness of the study, which may also account for this. Recruitment of eligible patients turned out to be a major challenge for the completion of the study, despite including multiple animal hospitals. The strict inclusion criterion of acute stroke (<72 h) with MRI verification is a clear hurdle to the enrolment of dogs as only a few veterinary hospitals have direct access to MRI.

Conclusion

Community-dwelling domestic dogs may have a place in stroke research as a supplement to existing experimental animal models. Although the 'spontaneous stroke dog model' faces several challenges, in particular with respect to the heterogeneity in animals (e.g. age, breed, stroke location, risk factors), which can be controlled in traditional animal models, this may, however, present a setting that resembles the very heterogeneous circumstances of humans. Striking similarities to human ischaemic stroke have reported previously been with respect to symptomatology and infarct topography [14,15]. In addition, the present study showed that IL-6 concentrations increased in the CSF and plasma of dogs with acute ischaemic stroke, which also corresponds well with the findings in humans, supporting the translational potential of spontaneous canine ischaemic stroke. Our results support the dog with spontaneous ischaemic stroke as an alternative clinical and noninvasive model for studies of early infarct-related pathophysiology. Because of the limited number of dogs investigated, we recommend that our results be further explored in a larger scale study.

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Authors' contribution: H.G., B.B.T., D.A., B.F., A.M., K.L.L. and M.B. conceived the study; H.G. and B.B.T. collected the clinical data, blood and CSF of the Danish

dogs, and interpreted results; H.G. furthermore carried out the statistical analysis, and drafting of the manuscript; L.G. and C.R. collected clinical data, blood and CSF of the British dogs; A.B.-S. and T.D. carried out the Mesoscale analyses; A.M. headed infarct estimations; K.L.L. further assisted with close supervision of laboratory processes and in the drafting of the manuscript together with D.A. and B.F.

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Conflicts of interest

There are no conflicts of interest.

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